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Comparison of the Efficacy of Enrofloxacin and Lactobacillus Plantarum Cell-Free Supernatant Treatments on Vaginitis in Ewes

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Abstract

The aim of the study was to investigate the effect of different intravaginal treatment strategies on the vaginal discharge score, vaginal microbiota, bacterial and Enterobacteriaceae counts in nulliparous Merino ewes. All ewes (n=45) received intravaginal sponges containing 60 mg medroxyprogesterone acetate for 7 days and allocated into three equal groups (n=15). Sponges were injected Lactobacillus plantarum cell-free supernatant (SUPER), enrofloxacin (ENRO), or physiologic saline (CON) prior to sponge insertion. At sponge removal, 500 IU equine chorionic gonadotropin (eCG) were administered in all ewes. For the detection of vaginal microbiota, bacterial and Enterobacteriaceae counts, samples were collected prior to sponge insertion, at sponge withdrawal, and 48 h later after sponge withdrawal. Vaginal discharge score was not different in ENRO (2.26±0.18) and SUPER (2.20±0.14) compared to CON (2.46±0.16). The time-dependent alteration was significant for the mean bacterial and Enterobacteriaceae count in all groups (P<0.05). Bacterial counts were found to be lower in ENRO (5.50±0.17) than SUPER (6.31±0.19) and CON (6.07±0.15) at sponge removal (P<0.05). In addition, SUPER (3.74±0.21) and ENRO (3.49±0.27) had lower Enterobacteriaceae counts compared to CON (4.78±0.21) at sponge removal (P<0.01). The most frequently isolated bacteria species were Trueperella pyogenes (28.9%) and Escherichia coli (46.7%). In conclusion, treatment with enrofloxacin or cell-free supernatant decreased the Enterobacteriaceae counts in ewes. Comprehensive studies are needed to assess the effectiveness of lactic acid bacteria as an antibiotic-free treatment strategy on vaginitis in ewes that were synchronized with progesterone impregnated intravaginal sponge.

Keywords: Antibiotic, Bacterial count, Lactobacillus plantarum, Sheep, Vaginitis

Introduction

Reproduction of small ruminants is commonly controlled by using the progesterone impregnated intravaginal sponges to achieve optimal lamb production.[1] Intravaginal sponge generates local inflammation with histological and cytological alterations in the vaginal flora.[2,3] Narrow vaginal lumen of nulliparous ewes increase the severity of vaginal inflammation compared to multiparous ewes.[4] These alterations are explained by the proliferation of pathogenic bacteria and the presence of abnormal mucus accumulation at sponge withdrawal.[3,5] In addition to vagina inflammation, the collection of mucus impaired sperm quality in rams[6] and decreased the sexual attractiveness of ewes to rams.[7] Therefore, vaginitis after using intravaginal sponges leads to the reduction of pregnancy rate.[8]

Vaginal bacterial load number increases with vaginitis at sponge removal compared to sponge insertion in ewes.[2] Escherichia coli, Staphylococcus spp., and Trueperella spp. constitutes the majority of the vaginal microbiota in ewes

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with vaginitis.[5,8–13] Efficient antibiotic therapy such as enrofloxacin was recommended to reduce the detrimental effects of vaginitis in recent studies.[8] However, controlling vaginitis by adding antibiotics into sponge has not been preferred due to the resistance of microorganisms[8,14] and drug residues.[15,16] Considering the resistance of microorganisms to antibiotics and residue for human health, antibiotic-free treatment options should be investigated.

Recent studies reported that Lactobacillus species were commonly used as antibiotic-free therapy to treat bacterial vaginosis in women.[17,18] These bacteria secrete many natural antimicrobial metabolites such as bacteriocin, phenyl lactic acid, organic acids, and hydrogen peroxide.[18] The inhibitory effect of lactic acid bacteria on pathogenic bacteria is likely associated with the antimicrobial compounds produced by lactic acid bacteria. However, Quereda et al. (2020)[9] reported that infusion of lactic acid bacteria did not decrease bacterial counts in ewes that had vaginitis. L. plantarum is one of the most effective probiotic agents that inhibit bacterial pathogens.[19,20] The objective of this study was to compare the effectiveness of L. plantarum cell-free supernatant or enrofloxacin on vaginal discharge score, vaginal microbiota, the vaginal bacterial load, and the number of Enterobacteriaceae in nulliparous ewes.

Materials and Methods

Animals and Management

The experimental procedures were approved by the Balıkesir University Animal Care Committee (Approval no: 2021/3-4). The study was carried out during non-breeding season (March-May) in a farm located in Bursa (40° 13' N, 29° 29' E) in Turkey. Forty-five nulliparous Merino ewes, 10-13 month age and body condition score of 3.0-3.5, were used in the study. Ewes were grazed on natural pasture and received an average 200 g alfaalfa (Medicago sativa) per ewe/day and 1000 g concentrate barley per ewe/day during the experiment. Ewes had ad libitum access to good-quality drinking water.

Experimental design

Intravaginal sponges containing 60 mg medroxyprogesterone acetate (MAP; Esponjavet®, HIPRA, Turkey) were inserted and remained in situ for 7 days in all ewes. Ewes were randomly divided into three groups (n=15 for each group) receiving two treatment groups and a control group as follows; L. plantarum cell-free supernatant (SUPER; 1 mL/sponge), Enrofloxacin (ENRO; 0.5 mL, 10%, Enrolen®, Alke, Turkey) diluted with 0.5 mL 0.9% NaCl (the solution was applied totally as 1 mL in each sponge) or Control (CON; 1 mL/sponge, 0.9%, NaCl, Polifarma®, Turkey). All applications were injected at six different points on the sponge after the sponge was placed on the applicator to prevent the overflow of solutions. All materials were disinfected with 1% benzalkonium chloride (Zefirol, Dermosept®, Turkey) before each application. Ewes received one intramuscular injection of 500 IU equine chorionic gonadotropin (eCG; Gonaser®, HIPRA, Turkey) at the time of sponge removal. Following the removal of sponge, oestrus detection was performed with five teaser rams with crayon marks at 12-h intervals for 5 days.

Preparation of cell-free supernatants and antimicrobial activity

L. plantarum (DSM 1055) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ®, Germany). L. plantarum strain was grown in De Man Rogosa and Sharpe Broth (MRS Broth®, Merck, Germany) media at 37° C for 48 h. Bacterial suspension was transferred to sterile 10 milliliters falcon tubes. Bacterial cells were removed by centrifugation at 5000 g for 10 min. Cell-free supernatant was concentrated by either 50% or 75% with evaporator (55-60 °C/0.75 atm) to increase the concentration of antimicrobial activity. Cell-free supernatant was obtain after concentrated supernatant was filtered through a 0.22µ filter. The cell free supernatant was prepared 2 days before the application of sponge.

The three different concentrations (0%, 50% and 75%) of cell-free supernatant from L. plantarum and enrofloxacin were assessed their antimicrobial capability against E. coli (American Type Culture Collection, ATCC 25922) and collection of coliform bacteria in the Department of Public Health in of Balıkesir University. Agar well diffusion assays were performed on 90 mm Petri dishes containing Mueller Hinton agar media (Merck, Germany).[21] The agar plates were cut for 4 mm depth to place 50 µl of supernatant or enrofloxacin into each well. The plates were incubated at 37 °C for 48 hours. Following incubation, the diameters of the growth inhibition zones were measured in millimeters to the nearest 0.1 mm using electronic callipers.[21] Each stage was repeated two times. For the antimicrobial activity of enrofloxacin and three concentrations of cell-free supernatant, the mean diameters of inhibition zones are presented in Table 1.

Table 1. Zone diameters of Lactobacillus plantarum cell-free supernatant (SUPER) at different concentrations and enrofloxacin (ENRO) against bacteria on Mueller Hinton Agar

Bacteria	SUPER	SUPER %50	SUPER %75	ENRO
Escherichia coli	20.66 ± 0.57	24.33 ± 0.57	33.00 ± 1.00	40.00 ± 1.00
Coliform bacteria	19.66 ± 0.57	23.33 ± 0.57	32.00 ± 1.00	41.33 ± 0.57

SUPER: *Lactobacillus plantarum* cell-free supernatant; ENRO: Enrofloxacin

Evaluation of vaginitis

Vaginitis mucus were scored according to vaginal discharge characteristics (amount and aspect) as follows; score 0: negligible or no discharge, score 1/moderate: clear and some amount of discharge, and score 2/severe: abundant purulent or hemorrhagic discharge.[22] The odour of vaginal discharge (score 0 = none, score 1 = mild, and score 2 = abundant) was determined at sponge removal according to Viñoles et al. (2011)[13] following the sponge removal, sponges were weighted to determine the accumulation of mucus secretion.2 Vaginal pH is determined using pH indicator strips (Merck®, Germany) at the sponge insertion and sponge removal.

Collection of Vaginal Samples

All samples were collected on the day of sponge insertion (day 0), at sponge removal (day 7), and 48 h later (day 9). The vulvar area of ewes was disinfected with 1% benzalkonium chloride (Zefirol®, Dermosept) and the vaginal lips were opened with one veterinarian to avoid contamination. Samples were collected using sterile stuart transport swab (Fıratmed®, Turkey) which was rotated over the anterior vaginal mucosa by direct contact for bacterial examination. For the counting of colony forming units (log CFU/mL) and Enterobacteriaceae, the second samples were collected using sterile plastic stick swab in the same technique as the bacterial samples and swab was placed in a 9 mL of Maximum Recovery Diluent (MRD, Merck®, Germany) at neutral pH. The samples were transported at 4°C to the laboratory after 2 h following collection.

Bacterial Count

Tubes, diluted in 9 mL of MRD at neutral pH, was homogenized by vortex for 1 min to suspend the bacteria. Serial dilutions were performed using MRD and dilutions were plated in Plate Count Agar (PCA, Merck®, Germany) for counting total bacterial count (CFU/mL). Dilutions were seeded on Violet Red Bile Dextrose agar medium (VRBD, Merck®, Germany) for Enterobacteriaceae count. Bacterial colonies were counted after incubation of PCA and VRBD agar for 48 h at 37°C.

Identification of Bacterial Population

Vagina swab samples were submitted to the microbiology laboratory and processed within 2 hours of retrieval form ewes. The samples were cultured for bacteria with 5% defibrinated sheep blood and incubated at 37°C. After incubation for 24-48h, plates were examined for growth. Colonies are stained with Gram stain. BBLTM CrystalTM biochemical tests were used according to manufacturers' instructions for identification of colonies.

Statistical Analysis

All statistical analyses were performed using SPSS® 23.0 package software (IBM Corporation, NY, USA). Total bacterial count (CFU/mL) and Enterobacteriaceae count were analyzed with analyses of variance (ANOVA) for repeated measurements after logarithmic transformation to normalize the data. Age, vaginal pH, vaginitis discharge score, vaginal odour score, and sponge weight were analyzed using ANOVA. Oestrus response and percentage of vaginal discharge score was analyzed using chi-square test Statistical significance level of $P<0.05$ was considered as significant, and statistical tendencies were defined as $0.05<P<0.10$.

Results

The mean age of ewes 11.84±0.48 month and there was no difference ($P>0.05$) for the age of ewes among the groups (CON; 11.72±0.83, ENRO; 11.76±0.91, and SUPER; 12.03±0.81) at the beginning of study. Oestrus response was 46.7% (21/45) and there was no significant difference among the groups, as follows CON; 53.3%, ENRO; 33.3% and SUPER; 53.3%. Vaginal discharge of varying severity was detected following the sponge removal in all ewes. The overall percentage of negligible discharge (Score=0; 8.9%, 4/45) was lower than moderate (Score=1; 51.1%, 23/45) and severe ones (Score=2; 40.0%, 18/45) irrespective of groups ($P<0.01$). The percentage of negligible, moderate and purulent/hemorrhagic vaginal discharge were 6.7%, 40.0%, 53.3% for CON, 13.3%, 46.7%, 40.0% for ENRO, and 6.7%, 66.6%, 26.7% for SUPER, respectively, data was shown at Table 2. When evaluated the mean vaginitis score among the groups, both of the two treatments (ENRO; 2.26±0.18 and SUPER; 2.20±0.14) did not affect ($P>0.05$) on the vaginitis score compared to that of CON (2.46±0.16).

The mean weight of the sponge at the time of removal was significantly heavier ($P<0.01$) in the ENRO (6.92±0.59 g) compared to those of SUPER (5.14±0.22 g) and CON (4.92±0.17 g). However, there was a tendency ($P=0.08$) for reduced odour score of vaginal discharge in ENRO

Table 2. Comparison of different parameters following different treatment with medroxyprogesterone acetate plus eCG in nulliparous ewes

Parameters	OVERALL	CON	ENRO	SUPER	*P*
Age (months)	11.84 ± 0.48	11.72 ± 0.83	11.76 ± 0.91	12.03 ± 0.81	>0.05
Vaginal discharge score 1 %, (n/n)	8.9% (4/45)	6.7% (1/15)	13.3% (2/15)	6.7% (1/15)	>0.05
Vaginal discharge score 2 %, (n/n)	51.1% (23/45)	40.0% (6/15)	46.7% (7/15)	66.7% (10/15)	>0.05
Vaginal discharge score 3 %, (n/n)	40.0% (18/45)	53.3% (8/15)	40.0% (6/15)	26.7% (4/15)	>0.05
Vaginal discharge score (mean ± S.E.M)	2.31 ± 0.09	2.46 ± 0.16	2.26 ± 0.18	2.20 ± 0.14	>0.05
Odour score of vaginal discharge (mean ± S.E.M)	2.18 ± 0.12	2.40 ± 0.19^{a}	1.80 ± 0.22^{b}	2.33 ± 0.19^{a}	0.08
pH at sponge insertion	7.00 ± 0.06	7.05 ± 0.06	7.03 ± 0.10	7.10 ± 0.11	>0.05
pH at sponge removal	7.21 ± 0.05	7.19 ± 0.63	7.18 ± 0.97	7.24 ± 0.90	>0.05
Sponge weight (g)	5.66 ± 0.25	4.92 ± 0.17^{a}	6.92 ± 0.59^{b}	5.14 ± 022^{a}	<0.01

^{a,b} Values within a row with different superscripts differ significantly at $^{**}P<0.01$; $^{*}0.05<P<0.10$ (tendency)

OVERALL: The mean value of all groups; CON: Control; ENRO: Enrofloxacin; SUPER: *Lactobacillus plantarum* cell-free supernatant

(1.80±0.22) compared to CON (2.40±0.19) and SUPER (2.33±0.09). The mean vaginal pH (mean±S.E.M) was not different (P>0.05) at sponge insertion (7.05±0.06, 7.03±0.10, 7.10±0.11) and following sponge removal (7.19±0.63, 7.18±0.97, 7.24±0.90) among the groups (CON, ENRO and SUPER, respectively).

Table 3. Comparison of total bacterial count (log CFU/mL) and Enterobactericea count (ENTERO) at sponge insertion (day 0), sponge removal (day 7), and 48 later after sponge removal (day 9) following different vaginal treatment protocol with synchronization eCG in ewes

Bacterial Count	OVERALL	CON	ENRO	SUPER	P
CFU at day 0	4.44 ± 0.14	4.60 ± 0.25	4.12 ± 0.20	4.58 ± 0.25	>0.05
CFU at day 7	5.96 ± 0.11	6.07 ± 0.15[a]	5.50 ± 0.17[b]	6.31 ± 0.19[a]	<0.05
CFU at day 9	5.32 ± 0.13	5.40 ± 0.23	5.17 ± 0.25	5.39 ± 0.20	>0.05
ENTERO at day 0	1.99 ± 0.13	2.03 ± 0.23	1.96 ± 0.24	1.98 ± 0.18	>0.05
ENTERO at day 7	4.00 ± 0.16	4.78 ± 0,21[a]	3.49 ± 0,27[b]	3.74 ± 0,21[b]	<0.01
ENTERO at day 9	3.35 ± 0.17	3.85 ± 0.30	3.04 ± 0.30	3.15 ± 0.26	>0.05

a,b Values within a row with different superscripts differ significantly at *P<0.05; **P<0.01;

OVERALL: The mean value of all groups; CON: Control; ENRO: Enrofloxacin; SUPER: Lactobacillus plantarum cell-free supernatant

The mean bacterial count (log CFU/mL) was 4.44±0.14 at sponge insertion regardless of groups. The mean bacterial count significantly increased (5.96±0.11) at sponge removal and it decreased (5.32±0.13) at 48 h later following the sponge removal (P<0.01). The log CFU/mL in ENRO (5.50±0.17) was lower compared to SUPER (6.31±0.19) and CON (6.07±0.15) at sponge removal (P<0.05). Similar to log CFU/mL, the time-dependent alteration was also significant in the number of Enterobacteriaceae (P<0.05). The mean value of the Enterobacteriaceae count was 1.99±0.13 at sponge insertion, 4.00±0.16 at sponge removal, and 3.35±0.17 at 48 later after sponge removal. The number of Enterobacteriaceae was not significant at sponge insertion and 48 h later after sponge removal among groups. However, the Enterobacteriaceae counts in SUPER (3.74±0.21) and ENRO (3.49±0.27) was lower (P<0.01) at sponge removal when compared to CON (4.78±0.21) group (Table 3). Additionally, the bacterial count and Enterobacteriaceae at two days later after sponge removal was not different (P>0.05) in ewes showed oestrus compared to ewes did not show oestrus.

The number of vaginal bacteria species isolated in different stages of synchronization was presented in Table 4. Irrespective of treatment groups, the number of Gram-positive bacteria was 7/45 (15.5%) of samples at sponge insertion, 35/45 (77.8%) at sponge removal, and 33/45 (73.3%) at 48 hours after sponge withdrawal. The number of Gram-negative bacteria was 4/45 (8.9%) of samples at sponge insertion, 22/45 (48.9%) at sponge removal, and 18/45 (40.0%) at 48 hours after sponge withdrawal. Irrespective of the groups and timing of sampling, 16 Gram-positive and 3 Gram-negative bacteria species were isolated. The most frequently isolated bacteria species were T. pyogenes (28.9%) and E. coli (46.7%) at sponge removal. For the experimental groups, the number of T. pyogenes was 4/15 (26.7%) for SUPER, 0/15 (0%) for ENRO, and 9/15 (60.0%) for CON at sponge removal. The number of E. coli was 11/15 (73.3%) for SUPER, 4/15 (26.7%) for ENRO, and 9/15 (40.0%) for CON at sponge removal.

Table 4. Distributions of vaginal bacteria on different sampling days according to different treatment strategies in nulliparous ewes

	SUPER			ENRO			CON		
	Days of sampling								
	0	7	9	0	7	9	0	7	9
Gram-positive bacteria									
Staphy scheleiferi	-	1/15	1/15	-	-	-	-	-	-
Enterococcus faecium	-	1/15	-	1/15	2/15	-	-	-	-
Enterococcus faecalis	-	-	-	-	1/15	1/15	-	-	-
Trueperella pyogenes	1/15	4/15	1/15	-	-	2/15	-	9/15	8/15
Streptecoccus acidominimus	-	1/15	-	-	2/15	2/15	-	-	-
Enterococcus durans	-	-	1/15	-	-	-	-	1/15	1/15
Staphylococcus xylosus	-	-	-	-	1/15	2/15	-	-	-
Streptecoccus uberis	1/15	2/15	1/15	1/15	2/15	1/15	-	1/15	1/15
Aerococcus viridans	-	-	-	-	-	-	-	1/15	-
Staphylococcus equorum	-	-	-	-	-	-	1/15	1/15	4/5
Staphylococcus kloosii	-	-	-	1/15	1/15	1/15	-	-	-
Streptecoccus pneumonie	-	-	-	-	1/15	1/15	-	-	-
Streptecoccus galloly ssp. galloly	-	1/15	-	1/15	1/15	1/15	-	-	-
Enterococcus hirae	-	-	1/15	-	-	-	-	-	-
Staphylococcus lentus	-	-	1/15	-	1/15	-	-	-	-
Staphylococcus gallinarum	-	-	-	-	-	-	-	-	1/15
Overall	2/15	10/15	6/15	4/15	12/15	11/15	1/15	13/15	15/15
Gram-negative bacteria									-
Escherichia coli	1/15	11/15	7/15	1/15	4/15	5/15	1/15	6/15	4/15
Proteus mirabilis	-	-	1/15	-	-	-	-	-	-
Alcaligenes faecalis	-	-	-	1/15	1/15	1/15	-	-	-
Overall	1/15	11/15	8/15	2/15	5/15	6/15	1/15	6/15	4/15

CON: Control; ENRO: Enrofloxacin; SUPER: *Lactobacillus plantarum* cell-free supernatant

Discussion and Conclusion

The vaginal discharge rate was 100% in nulliparous ewes as a response of vaginal mucosa to the presence of the sponge in this study. The increment of vaginal discharge rate could resulted from narrow vaginal lumen of nulliparous ewes[4]. Previous studies reported that vaginal discharge rate was varied from 98.5% to 100% after use of progesterone impregnated intravaginal sponge in multiparous ewes.[22,23] Local treatment with tetracycline and penicillin seems to be the widespread choice rather than enrofloxacin in previous studies.[12,13] However, enrofloxacin was reported as the most effective antibiotic to control the vaginitis due to high sensitivity to vaginal microorganism in a recent study.8 Although most of previous studies reported the treatment strategies of vaginitis with antibiotics, vaginal discharge score was not evaluated.[5,8,13,24,25]

The severity of vaginal discharge after sponge removal did not differ among groups in this study. Additionally, the bacterial count of vaginal flora was not different prior to sponge insertion among three groups. The presence of

sponge as a foreign body and bacterial load causes the infiltration of leucocytes into the vagina.[26] Similar to previous studies.[5,26] bacterial count significantly increased at sponge removal in all groups in the present study. The increment of bacterial counts at sponge removal was significantly lower in ENRO group than other groups. Besides, this benefit was consistent with decreased the odour of vaginal discharge in ENRO compared to other groups. Viñoles et al. (2011)[13] reported the decreased odour of sponges after spraying sponges with antibiotics. According to our observation, mucus accumulation was more fluid in ENRO and this effect may have resulted in a higher sponge weight in ENRO. Our results was consistent with the previous studies which recommended fluoroquinolones such as enrofloxacin[8] and ciprofloxacin[10,25] to control the vaginitis in ewes.

Several studies reported that original microbiota at sponge insertion was recovered at 48 h later after sponge removal.[2,5,27] Bacterial count decreased at 48 h later after sponge removal in all groups compared to that of sponge removal. However, bacterial count at 48 h later after sponge removal did not return similar values at sponge insertion in this study.[8,11] Bacterial count at 48 h later after sponge removal is significant, as ewes show oestrus at that time and they mate with rams.[28] Furthermore, overall oestrus detection rate was 46.7% and bacterial count at two days later after sponge removal was not changed depending on oestrus expression in all groups. Previous studies indicated that local vaginal conditions vary depending on not only different stages of the oestrus cycle but also vaginal bacterial species.[11,29]

The predominant bacteria species were T. pyogenes and E. coli in this study. These results are in agreement with those isolated bacteria related to vaginitis in ewes in previous studies.[11,13,29] Continual contamination of the sponge string by faeces, urine and the retention of intravaginal foreign bodies leads to higher isolation of T. pyogenes and E. coli in vaginal mucus.[13] These important pathogens cause the severe vaginal discharge, suppression of estradiol production.[29] When comparing the previous studies[4,28], the lower oestrus reponse could be resulted from the presence of T. pyogenes and E. coli in this study. Consistent with the our results, many studies reported that the mostly isolated dominant pathogen was Enterobacteriaceae at sponge removal.[8,9,12,26] The number of Enterobacteriaceae at sponge removal was lower in ENRO compared to that in CON in this study. Enrofloxacin inhibits the bacterial DNA gyrase and is more effective on Gram-negative bacteria.[8] The reduction in bacterial counts mainly could have resulted from the high sensitivity to enrofloxacin of vaginal bacteria in this study.[30] Antibiotic resistance and residue is a major challenge to control vaginitis in ewes.[15,25] For this reason, lactic acid bacteria or their cell-free supernatant was a plausible strategy to control vaginitis by preventing the high vaginal pH which is required for pathogenic bacteria to growth.[9] Lactic acid bacteria are well known as natural preservatives for their antimicrobial activity against pathogens in foods.31 Similarly, previous reports revealed the potential beneficial effects of lactic acid bacteria in cows with reproductive tract infections.[32,33]

Quereda et al. (2020)[9] firstly reported the efficacy of commercial probiotic bacteria mix of 60% Lactobacillus crispatus, 20% Lactobacillus brevis and 20% Lactobacillus gasseri on vaginitis in ewes. The authors reported that the use of lactic acid bacteria had no beneficial effect on bacterial counts and the number of Enterobacteriaceae. Consistent with previous study[9], the administration of cell-free supernatant of L. plantarum prior to sponge insertion did not change the bacterial count in this study. However, the use of cell-free supernatant significantly reduced the number of Enterobacteriaceae at sponge removal. In aggreement with previous report in cows[33], the use of cell-free supernatant of lactic acid bacteria as antimicrobial substances had a beneficial effect on Enterobacteriaceae in ewes that had vaginitis after using progesterone impregnated intravaginal sponge.

In conclusion, progesterone impregnated intravaginal sponge insertion leads to vaginal discharge and increased bacterial count in all ewes. The most frequent bacteria were T. pyogenes and E. coli. The addition of enrofloxacin into sponge reduced the total bacterial and Enterobacteriaceae count. Cell-free supernatant was effective for reducing the Enterobacteriaceae count. Normal vaginal flora composition partially recovered at 48 h after sponge removal in all groups. As an alternative strategy for the use of antibiotics, further studies are needed to evaluate the effectiveness of antimicrobial substances (cell-free supernatant) on vaginitis in ewes.

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